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Orphan chemokine receptors in neuroimmunology

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Identification of orphan chemokine receptor HCR in human astrocytes as the human homologue of L-CCR: functional similarities

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Submitted

Summary

We have recently characterized the murine orphan chemokine receptor L-CCR in mouse glial cells. In the current study we have examined the mRNA and protein expression of a human orphan chemokine receptor HCR in cultured astrocytes. Furthermore, functional studies in the human embryo kidney (HEK293) celline transfected with HCR indicated candidate ligands for HCR. Chemotaxis assays, intracellular calcium measurements and filamentous actin staining showed significant responses of HCR transfected HEK293 cells to the CC chemokines CCL2, CCL5, CCL7 and CCL8. Based on the similarities between the DNA sequence, protein sequence, and functional properties of L-CCR and HCR, it is proposed that HCR is the human homologue of the mouse orphan chemokine-like receptor L-CCR.

Introduction

The activation and homing of immune cells during an inflammatory response is orchestrated largely by chemotactic cytokines (chemokines). The chemokine family consists of small (approximately 10kDa) proteins that bind to G-protein coupled chemokine receptors. Based on the localization of conserved cysteine residues in their molecular structure, chemokines are divided into 4 subfamilies: C, CC, CXC and CX3C. Currently, more than 25 CC human chemokines and 10 CC human chemokine receptors (CCR) have been cloned (25). Most chemokines can act on more than one chemokine receptor and most chemokine receptors can be activated by different chemokines. Although chemokines were originally detected in the peripheral immune system, recent years have shown expression of chemokines and receptors in the central nervous system (CNS). Thus, there is evidence of chemokine and chemokine receptor involvement in brain pathology (for reviews see 3-4, 9, 16, 26). The cellular localization of chemokines and their receptors in the CNS is a subject of investigation. A number of reports have described CC chemokine receptor expression in astrocytes. Specifically, CCR1 and CCR5 expression was shown in astrocytes (1, 14, 23). The appearance of contradictory reports makes the expression of CCR2 in astrocytes a controversial subject (see for example 2, 19, 35). In addition, at least 4 orphan chemokine receptors have been found expressed in astrocytes (14, 18, 27, 35). We have recently shown expression of the orphan receptor lipopolysaccharide (LPS) -inducible CC chemokine receptor (L-CCR) in murine astrocytes *in vitro* and *in vivo* (35). In addition, L-CCR expression was regulated under pro-inflammatory conditions. Furthermore, we have reported a number of endogenously expressed candidate ligands for L-CCR (10). In the current study, we have identified the human orphan chemokine receptor HCR as a possible human homologue of L-CCR.

Materials and Methods

Materials

We used Hank's balanced salt solution, HAMF10 and Dulbecco's Modified Eagle's Medium from Gibco (Life Technologies), porcine trypsin, poly-L-lysine and TRITC-Phalloidin from Sigma, oligonucleotides for polymerase chain reactions from Genset Oligos, pCR2.1 and pcDNA3.1 (-) vectors from Invitrogen, Fugene® 6 Transfection Reagent from Roche, G418 selection antibiotic from Calbiochem, the leakage resistant calcium indicator Fura-PE3(AM) from Teflabs, Vectashield® protective mounting fluid from Vectalabs, and all recombinant human chemokines and the HCR antibody from R&D Systems®. Standard chemicals were obtained through Merck, Sigma and Gibco. Equipment that was used: chemotaxis microchamber from Neuroprobe, Photometrix SensiCam CCD camera, Leica TCS S2 / TM RXE Confocal Laser Microscope set.

Cell cultures

Human astrocyte cell cultures were established as described previously (13). In brief, tissue samples from post mortem adult subcortical white matter or cortex were collected and meninges and visible blood vessels were removed after which the tissue was minced into cubes of $\leq 2 \text{ mm}^3$. The tissue fragments were then incubated at 37°C for 20 minutes in a Hank's balanced salt solution containing 0.25% w/v porcine trypsin (Sigma, St. Louis, MO), 0.2 mg/ml EDTA, 1 mg/ml glucose and 0.1 mg/ml bovine pancreatic DNase I. After digestion cell suspensions were gently triturated, washed and taken into culture in Dulbecco's Modified Eagle's Medium (high glucose; Life Technologies, Breda, The Netherlands) mixed 1 : 1 with L-glutamine containing HAM-F10 (Life Technologies, Breda, The Netherlands) and supplemented with 10% fetal calf serum and 0,01% penicillin/0.01 streptomycin in a humidified atmosphere (5% CO_2) at 37°C . To remove possible contamination of astrocyte cultures with meningeal and blood monocyte derived macrophages, cell suspensions were grown overnight in uncoated tissue flasks allowing monocytes/macrophages to adhere. The supernatant was then transferred to poly-L-lysine (15 mg/ml, Sigma)-coated 80 cm^2 flasks and after 48h the medium was changed to remove unattached cells and myelin debris. These cultures contained 100% astrocytes (GFAP positive cells). Culture medium was refreshed every six days. Cells were passaged by rinsing the cells with PBS, incubation for 5-10 minutes with 0.2% trypsin at 37°C , washing with culture medium and replating the cells in 25 cm^2 flasks. Cells were used at early passage and not after passage 8.

Human microglial cell cultures were obtained as described previously (12) Proliferation of microglia was induced by addition of recombinant granulocyte-macrophage colony stimulating factor (recombinant human GM-CSF, Leucomax, Novartis, The Netherlands) to the adherent cell cultures every 3 days in a final concentration of 25 ng/ml. The culture medium was refreshed once a week. Adherent cells were passaged by harvesting the cells using a rubber cell scraper (Costar, MA, USA) or by trypsinization with 0.25% w/v porcine trypsin at 37°C . Human monocytes were kindly provided by the Department of Haematology of the Academic Hospital of Groningen (AZG), The Netherlands. Finally, HEK293 cells were maintained in DMEM containing 10% fetal calf serum with 0,01% penicillin and 0,01% streptomycin in a humidified atmosphere (5% CO_2) at 37°C .

Reverse transcripts polymerase chain reaction (RT-PCR)

Cells were lysed in guanidinium isothiocyanate/mercaptoethanol buffer and total RNA was extracted with slight modifications according to Chomczynski and Sacchi (11).

A) Reverse transcription: $1 \mu\text{g}$ of total RNA was transcribed into cDNA as described (8).

For potential contamination by genomic DNA was checked by running the reactions (35 cycles) without reverse transcriptase and using GAPDH primers in subsequent PCR amplifications. Only RNA samples, which showed no bands after that procedure, were used for further investigation.

B) Polymerase chain reaction: 2 μ l of the RT-reaction was used in subsequent PCR amplification as described (8). Primers for 361 basepair (bp) fragments of HCR were : 5'-GAGGCAGAGCAATGTGACAA-3' (forward primer) and 5'- TCCTCCTG GCTGAGAAAAAG-3' (backward primer) at 56 °C and with 32 cycles. Primers for 351 bp fragments of human CCR2 were 5'- TGGCTGTGTTTGCTTCTGTC-3' (forward primer) and 5'-CCAGTTGACTGGTGCTTTCA-3'. The housekeeping gene glycer-aldehyde-3-phosphate dehydrogenase (GAPDH) was primed for a 342 bp fragment with 5'- CATCCTGCACCACCAACTGCTTAG-3' (forward primer) and 5'- GCCTGCTTCACCACCTTCTTGATG-3' (backward primer) at 60°C and with 28 cycles.

All oligos were obtained from GenSet Oligos. Cloning into PCR2.1 (Invitrogen) and subsequent sequencing confirmed the correct identity of all PCR products.

Cloning and expression of HCR in HEK293

Primers to amplify the full-length sequence for HCR have been chosen according the intronless sequence for HCR (GenBank/EMBL Accession number: U97123). The full-length HCR coding sequence was amplified from human gDNA with the following primers: forward, 5'-AGTCTGAAGATGGCCAATTA-3'; backward 5'-CTTGCAATTTGGTG-GATGCTA-3'. The resulting PCR product was cloned in pCR2.1 (Invitrogen) for sequencing and subcloned into the *Bam*HI - *Not* I sites of pcDNA 3.1 (Invitrogen) for transfection. 1 μ g of the plasmid was transfected with 6 ml Fugene® (Roche Molecular Biochemicals) in HEK293 cells according to the manufacturer's instructions. Stable transfected cells were selected with G418 500 μ g/ml for approx. 2 weeks and the resulting cell clones were checked by RT-PCR for HCR mRNA expression. MOCK transfections were performed with either empty or enhanced green fluorescence protein (EGFP) containing pcDNA 3.1 expression vectors.

Determination of intracellular calcium

For calcium measurements, HCR-transfected HEK293 cells were cultured on glass coverslips and calcium measurements were performed as described earlier (7). Cells were loaded with Fura-PE3 (AM) for 1 hour. Fluorometric measurements were performed using a Photometrix SensiCam CCD camera supported by Axolab^R 2.1 imaging software. Digital images of the cells were obtained at an emission wavelength of 510 nm following paired exposures to 340 and 380 nm excitation wavelengths sampled at a frequency of 1 Hz. Fluorescence values representing spatial averages from a defined pixel area were recorded on-line. Increases in intracellular calcium concentrations were expressed as the 340/380 ratio of the emission wavelengths. Compounds were administered using a pipette positioned at a distance of 100-300 mm from the cells. All measurements were performed in absence of extracellular calcium.

Chemotaxis assays

Cell migration in response to chemokines was assessed using a 48-well chemotaxis microchamber (NeuroProbe^R). Chemokine stock solutions were prepared in PBS and further diluted in medium for use in the assay. Culture medium without chemokines served as a control in the assay. 27 μ l of the chemoattractant solution or control medium was added to the lower wells, lower and upper well were separated by a polyvinylpyrrolidone-free polycarbonate filter (8 μ m pore size) and 30000 cells per 50 μ l were used in the assay. Determinations were done in hexaplicate. The chamber was incubated at 37°C/5% CO₂ in a humidified atmosphere for 120 min. At the end of incubation the cells in the upper chamber were removed and residual cells on the filter were scraped off gently using a wet cottontip. The filter was then washed, fixed in methanol and stained with toluidine blue. Migrated cells were counted with a scored eyepiece (3 fields (1 mm²) per well) and migrated cells per chamber were calculated. The data are presented as mean values \pm S.D. and were analyzed by Students *t*-test. P values \leq 0.01 were considered significant.

F-Actin staining

Recombinant HEK293 cells, transfected with MOCK or HCR expression vector, and human astrocytes were cultured on glass coverslips in 6-well chambers. Prior to the experiment the medium was refreshed. CCL2 (R&D Systems) diluted in PBS was added to the cell cultures to an end concentration of 1×10^{-8} M. After set time intervals of 0 s, 30 s, 1 min, and 5 minutes the medium was swiftly removed and the cells were fixed in 4% PFA for 10 minutes. Subsequently, the cell-containing glass coverslips were washed twice for 10 minutes in PBS, followed by a 10 minute wash step in PBS + 0.1% Triton. Again, the glass coverslips were washed twice for 10 minutes in PBS after which they were incubated for 30 minutes in PBS containing 0.1 M TRITC-phalloidin (Sigma). The coverslips were then washed in PBS and mounted onto glass slides using Vectashield (VectorLabs, UK) for confocal fluorescence microscopy analysis.

Results

Identification of a possible human homologue of L-CCR

Previously, the human orphan chemokine receptor CKRX was proposed as a possible human homologue for L-CCR (29). The protein sequence of CKRX was for 51% identical to the murine L-CCR, whereas the murine chemokine receptor that showed highest homology to L-CCR was CCR3 with 36% identical aminoacids. In our search for the human counterpart of L-CCR we found 4 DNA sequences encoding the same human orphan chemokine receptor. These sequences are enlisted in the GenBank as HCR, CRAMA/B and CKRX. The DNA sequences of HCR, CRAM-B and CKRX are identical, whereas the sequence of CRAM-A contains 47 additional nucleotides at the 5' side.

Overall, the DNA sequences are 68% homologous to the L-CCR DNA sequence. Other genes listed in GenBank showed less than 57% homology in DNA sequence to L-CCR. Alignment of the protein sequences of HCR and L-CCR reproduced the 51% aminoacid homology Shimada and coworkers observed (Figure 5.1). Thus, HCR was investigated as a possible candidate human L-CCR-homologue.

HCR	MANYTLAPDEYDVL IEGELESDEAEQCDDKYDAQALVQPSLCFTSAVFI	IGVLDNLL	60
L-CCR	MDNYTVAPDDEYDVL I LDDY - LDNSGPDQVPAPFELSPQQVLQFCCFTAVFAVGLLDNVL		59
	* * * * * : * * * * * : * * * * * : * * * * * : * * * * * : * * * * * : * * * * *		
HCR	VVL I LVKYKGLKRMENIYLLNLAVSNLCFLLTLPFWAHAGGDP	MCK - FT - - - - I LIGLY	114
L-CCR	AVF I LVKYKGLKNMGN IYFLNLALSNLCLLPLPFWAHTAAHG	ESPGFTNGTCKVLVGLH	119
	. * : * * * * * * * * * * : * * * * * : * * * * * : * * * * * : * * * * * : * * * * *		
HCR	FVGLYSETFFNCLLTVQRYLVFLHKGNEFSARRRVPCGI I	TSVLAWVTAI LATFTLPEFV	174
L-CCR	SSGLYSEVFSN I LLLVQGYRVFSQ - GRLAS I FTTVSCGI	VAC I LAWAMAFTTALS	178
	***** . * * * * * * * * * * : . * : * * * * * : : * * * * * : * * * * * : * * * * *		
HCR	VYKPMEDQKYKCAFSRTPFLPADETFWKHFLT LKMNI	SVLVLPFI FTFLYVQFTMRKT	234
L-CCR	FYEPRMERQKHKCAFGKPHFLP I EAPLWKYVLTSKMI I	LVLAFFLLVFI I FTCCRQLRRR	238
	. * : * * * * * : * * * * * : . * * * * * : . * * * * * : * * * * * : * * * * * : * * * * *		
HCR	LRFREQRYSFLKLVFAVMVFLLMWAPYNI	AFFLSTFKEHFSLS	294
L-CCR	QSFREYQYDLHKPALV I TGVFLMWAPYNTVLFLSAFQEHL	SLQDEKSSYHFTLDASVQV	298
	* * * * * : . * . * * : : * * * * * * * * * * : * * * * * : * * * * * : * * * * * : * * * * *		
HCR	TKL I ATTHCCINPLLYAFLDG - TFSKYL	CRCFHLRSNTPLQPRGQSAQGT	353
L-CCR	TQLVATTHCCVNP LLYLLLDKAFMRYLRSLFPRCND I	PYQSSGGYQQAPPRFTEGHGRP	358
	* : * * * * * : * * * * * : * * * * * : * * * * * : * * * * * : * * * * * : * * * * *		
HCR	TEV - - - - -		356
L-CCR	I ELYSNLHQRQDI I		372
	* *		

Figure 5.1 ClustalW Protein sequence alignment of human HCR with the protein sequence of murine L-CCR showing that 47% of the protein sequence of HCR (GenBank/EMBL Accession number: U97123) is identical to L-CCR (GenBank/EMBL Accession number: AB009384) as indicated by asterixes. The following symbols denote the degree of conservation observed in each column: "*" means that the residues in that column are identical in all sequences in the alignment. ":" means that conserved substitutions have been observed. "." means that semi-conserved substitutions are observed.

HCR and CCR2 mRNA expression in human microglia and astrocytes

As L-CCR mRNA was originally detected in macrophages (29) and since our previous research showed expression of L-CCR mRNA in mouse microglia and astrocytes (35) we investigated expression of HCR mRNA in cultured human microglia and astrocytes using RT-PCR analysis. In addition, we examined human CCR2 expression in both cell types. HCR mRNA expression was detected in both cultured human microglia (Figure 5.2A) and astrocytes (Figure 5.2B), whereas CCR2 was only detected in human microglia (Figure 5.2A). Stimulation with lipopolysaccharide (LPS) upregulated HCR mRNA in human microglia, but not in human astrocytes (data not shown). Expression of HCR mRNA was upregulated, however, in human astrocytes by stimulation with a nitric oxide (NO) donor (Figure 5.2). The strongest expression of HCR mRNA was observed in astrocytes stimulated with 10^{-4} M of the NO-donor detanonoate. We found no mRNA expression of CCR2 in human astrocytes under control, LPS or NO-stimulated conditions (data not shown).

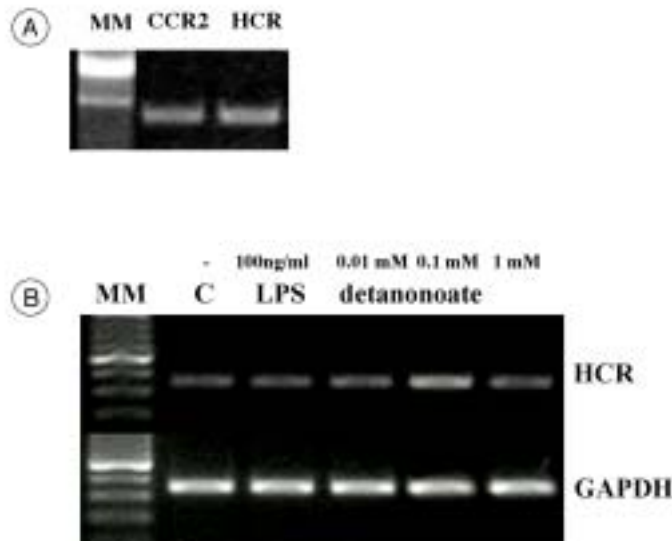


Figure 5.2 RT-PCR analysis of HCR mRNA expression isolated from human microglia and astrocytes. A. Microglia showed constitutive expression of CCR2 and HCR. B. Astrocytes showed constitutive expression of HCR. In addition, astrocytes were stimulated with lipopolysaccharide (100 ng/ml) or detanonoate (0.01, 0.1 and 1 mM). HCR mRNA expression was increased in 0.1 M detanonoate stimulated astrocytes compared to stimulation with other concentrations of detanonoate or unstimulated astrocytes. LPS did not alter HCR mRNA expression in astrocytes. Number of cycles for GAPDH and HCR were 28 and 32 respectively. MM, molecular weight marker, highlighted band is 500bp.

HCR Protein expression

In collaboration with R&D systems® an antibody directed to HCR was obtained that was used in immunocytochemistry assays. Standard lightmicroscopical immunohistological images of HEK293 cells transiently transfected with HCR showed protein expression of HCR (Figure 5.3). The cells were spotted on glass coverslips using a cytopspin assay. Control stainings without the primary antibody against HCR, showed only unspecific background staining (Figure 5.3A). In comparison, protein staining was strong in the presence of the primary antibody, with strong staining in the membrane area of the cell (DAB-staining in Figure 5.3B and fluorescent Cy3 staining in Figure 5.3C). Immunocytochemistry in cultured human astrocytes showed HCR protein expression (Figure 5.4B) that was upregulated after pre-stimulation of the astrocytes with 10^{-4} M NO for 24h (Figure 5.4C). Unspecific background staining in astrocytes was observed in absence of the primary antibody (Figure 5.4A).

HCR expression has previously been reported in cells of the monocytic lineage and this expression could be upregulated after LPS stimulation (24). We therefore tested the HCR antibody on human monocytes (Figure 5.5). When monocytes were stimulated with LPS (1 μ g/ml) for 24h a strong increase in immunofluorescence was detected and this increase was highest in the membrane area of the cells (Figure 5.5B). Control monocytes showed low levels of immunofluorescence (Figure 5.5A). The clustered staining of HCR near the cellular membrane in monocytes is similar to the staining of other known chemokine receptors, for example CXCR4 (Rey et al., 2002).

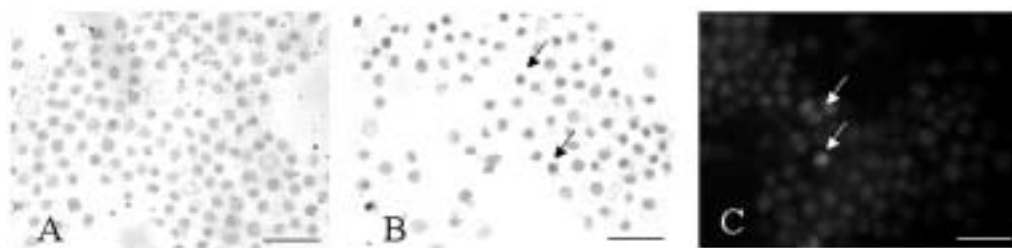


Figure 5.3 Light microscopic images of HCR immunocytochemistry with cytopspins of transiently HCR-transfected human embryo kidney cells, HEK293 (HCR) A.control (MOCK-transfected) HEK293 cells, DAB staining; B. HEK293(HCR) cells, DAB staining; C. HEK293(HCR), Cy3 staining. The arrows indicate examples of HCR positive cells. Space bars represent 100 μ m.

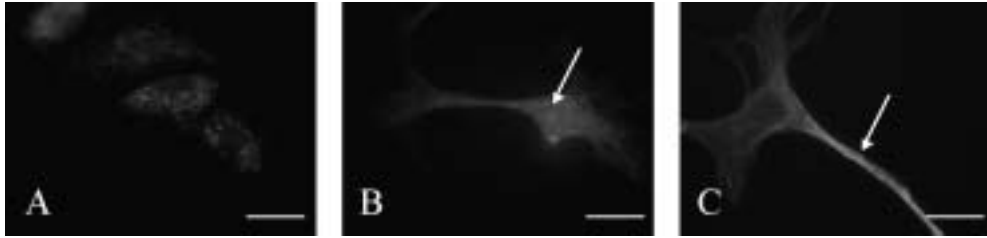


Figure 5.4 Confocal images of immunocytochemistry in cultured human astrocytes with an antibody against HCR, visualized with TRITC. A. the TRITC secondary antibody signal without the primary anti-HCR in control cells. B. TRITC-HCR signal in control astrocytes. C. TRITC-HCR signal in astrocytes stimulated with 10^{-4} detanonoate. Arrows indicate the HCR signal. Space bars represent 30 μm .

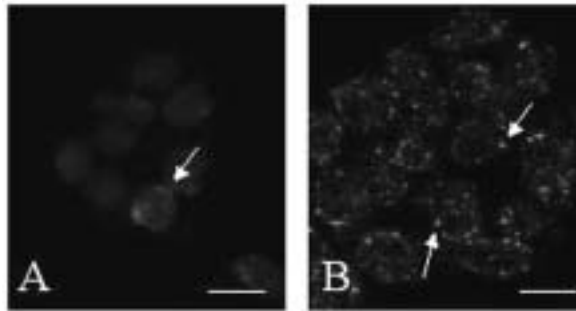


Figure 5.5 Confocal images of HCR-GFP immunohistochemistry with human monocytes incubated with (B) or without (A) LPS (1 $\mu\text{g}/\text{ml}$) for 24 h. Arrows indicate examples of the HCR protein signal. Note the much stronger HCR signal in LPS stimulated monocytes and the higher signal in the membrane of the cells. Space bars represent 50 μm .

Chemotactic responses to chemokines of HCR-transfected HEK293 cells

One key property of chemokine receptors is their ability to activate migration of cells in the direction of a chemokine concentration. We investigated migratory behaviour of HEK293 cells transiently transfected with or stably expressing HCR in response to a number of chemokines. We observed migration of HCR transfected HEK293 cells towards different concentrations of CCL2, CCL5, CCL7, CCL8, but not to CCL4, XC3CL1, XCL1, CXCL8, CXCL9 and CXCL12 (Table 5.1). In all chemotaxis experiments significance of migration was calculated using a one-tailed Student's *t*-test on the data. MOCK-transfected HEK293 cells showed only background migration in response to each chemokine tested (data not shown).

Table 5.1 Chemotaxis assays with HEK293 cells transiently transfected with HCR. Cells were stimulated in separate experiments with 10 different chemokines, in each experiment in a 10 nM concentration. The number of cells that migrated are expressed as a percentage of the background migration of unstimulated cells, with the corresponding SEM values. Asterixes indicate significant migration, when compared to background migration after a one-tailed Student's *t*-test with *n*=6.

Chemokine	Migration (% of control \pm SEM)
CCL2	177 \pm 11*
CCL5	180 \pm 10*
CCL7	147 \pm 18*
CCL8	161 \pm 15*
CCL4	112 \pm 13
XC3CL1	98 \pm 6
XCL1	110 \pm 5
CXCL8	78 \pm 15
CXCL9	102 \pm 10
CXCL12	132 \pm 8

Intracellular calcium increases in response to chemokines CCL5 and CCL2

It is known that chemokine receptors transfected in HEK293 cells induce rapid increases in intracellular calcium upon activation by chemokines (31). We therefore performed recordings of intracellular calcium transients in HEK293 cells transfected with HCR. Maximum detectable increases in intracellular calcium were observed after stimulation of the cells with 100 nM CCL5 (0.2 rise in 340/380 nm ratio on average, Figure 6A) and 100 nM CCL2 (0.06 rise in 340/380 nm ratio on average, Figure 5.6B). Lower concentrations of the chemokines only showed effects in few cells. Up to 1 μ M of other chemokines tested (CCL3, CCL4, CXCL9, CXCL10) did not induce intracellular calcium signals in HCR transfected HEK293 cells (Figure 5.6C). 100 μ M carbachol (D) was used in each experiment as a positive control (0.15 rise in 340/380 nm ratio on average, Figure 5.6D). 50 percent of the transiently transfected cells showed a strong response to CCL5 (12 \pm 2 out of 20 cells per experiment, *n*=4) and 30 percent of the cells responded to CCL2 (7 \pm 1 out of 20 cells per experiment, *n*=4). Interestingly, HEK293 cells transfected with HCR responded more strongly to CCL5 than to CCL2. None of the chemokines used induced intracellular calcium signals in MOCK-transfected HEK293 cells (0 cells out of 20 cells per experiment).

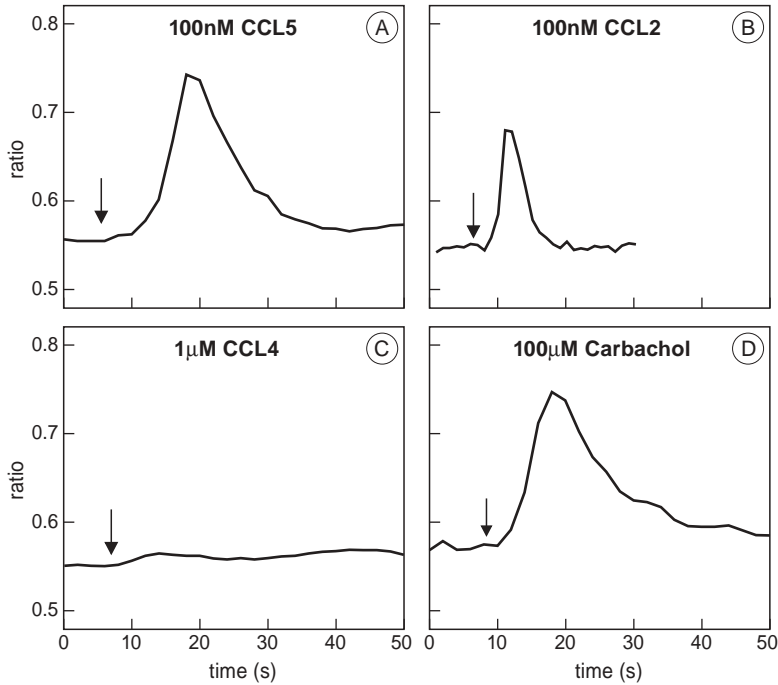


Figure 5.6 Intracellular calcium measurements in human embryo kidney cells (HEK293) stably transfected with HCR. The cells were stimulated with A. 100nM recombinant human (rh) CCL5, B. 100 nM CCL2, C. 1 μ M rhCCL4. Carbachol was used as a positive control (an average increase in 340/380 ratio with 0.15, D). The arrows indicate the moment of stimulus. Increases in intracellular calcium were seen in approximately 50 percent of the cells after stimulation with human CCL5 (an average increase in 340/380 ratio with 0.2), with a smaller increase in approximately 30 percent of the cells after CCL2 stimulation (an average increase in 340/380 ratio with 0.06). Cells did not respond to CCL4. The experiments were repeated 4 times.

F-actin distribution

One of the known properties of cells preparing for migration or responding to chemoattractants is the redistribution of filamentous actin (F-actin) in the cell (15, 30). Thus, F-actin shows a swift localization of the molecule at filopodia-like and lamellipodia-like structures at the cellular membrane, leading to polarization of F-actin. We used HEK293 cells transfected with either HCR or MOCK and stimulated the cells briefly (0 s, 30 s, 1 min and 5 min) with 10^{-8} M CCL2. We then stained the cells for F-actin. Fluorescent confocal microscopy revealed rapid redistribution and strong clustering of F-actin in HCR recombinant HEK293 cells after 30 seconds of stimulation (Figure 5.7C) and extending to later time points examined (data not shown). Unstimulated HCR transfected cells or MOCK transfected cells did not show this strong localization of F-actin after 30s (Figures 5.7A and B respectively) or any other timepoint examined.

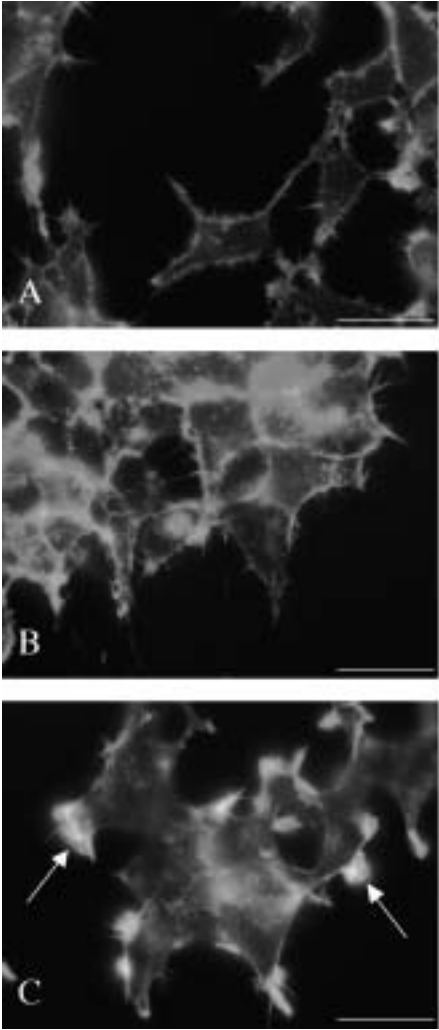


Figure 5.7 Confocal images of F-actin distribution in HEK293 cells by phalloidin-TRITC staining. Cells were transfected with either MOCK (A) or HCR (B and C). Cells in A and C were incubated for 30 seconds with CCL2. The arrows in C indicate examples of the strong polarization in F-actin distribution in cells transfected with HCR and incubated with CCL2, that is not present in non-stimulated HCR transfected cells or in cells incubated With CCL2 but transfected with MOCK. Space bars represents 50 μm .

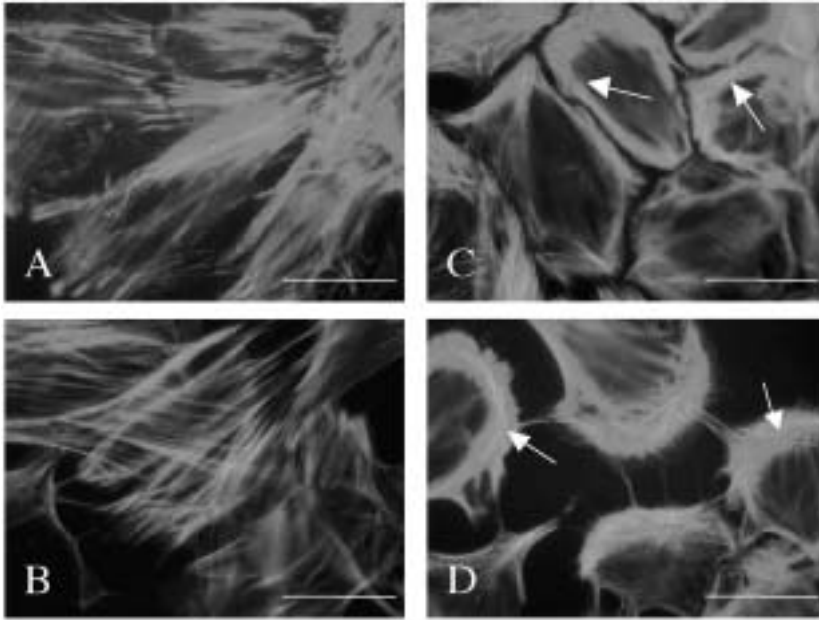


Figure 5.8 Confocal images of F-actin distribution in human astrocytes by phalloidin-TRITC staining. Cells were treated with 1×10^{-8} M CCL2 for 0 seconds (untreated, A), 30 seconds (B), 1 minute (C) and 5 minutes (D). After 30 seconds, the number of cells that show long parallel actin fibers decrease to an extent when compared to the untreated cells. However, after 1 minute, the astrocytes rearrange their F-actin distribution and start to show a coronal rim of F-actin, as well as deassociation with other astrocytes. After 5 minutes, this phenomenon is present in more than 50% of the cells. The arrows indicate examples of the strong coronal F-actin distribution in cells. Space bars represents 50 μm .

Similar experiments were also performed in cultured human astrocytes. Human astrocytes were treated with $10 \pm$ M CCL2 for 0 s, 30 s, 1 minute and 5 minutes. Untreated cells (0s) showed a high level of actin organization in elongated parallel fibers, overlapping multiple astrocytes (Figure 5.8A). 30 seconds after incubation with CCL2 the parallel fiber organization was impaired (Figure 5.8B). After 1 minute of incubation with CCL2 the parallel fiber organization was absent in $>40\%$ of the astrocytes and in $>60\%$ after 5 minutes. Instead, astrocytes showed less confluency and attachment to neighboring astrocytes (Figure 5.8C and 5.8D). In addition, they showed cortical rims of F-actin, identical to those seen in freshly plated astrocytes (17). The rearrangement of F-actin may also end in polarization of actin in the cell (Figure 5.8D). Together, these results showed rapid and dramatic actin reorganization in HCR-transfected HEK293 cells and human astrocytes in response to CCL2.

Discussion

We have previously characterized the orphan chemokine receptor, L-CCR, in murine glial cells *in vitro* and *in vivo* (35). Accordingly, the possible existence of a human homologue of L-CCR in glial cells was investigated. Shimada and colleagues (29) identified the human orphan chemokine receptor CKRX as a possible candidate L-CCR homologue and our subsequent sequence homology searches revealed that the sequence for CKRX is also listed in the GenBank database as CRAM and HCR. In the present study, we have investigated HCR localization and regulation in cultured post mortem adult human astrocytes and microglia. Although the microglia expressed HCR mRNA, we discarded microglia for further functional CCL2 studies as we also observed human CCR2 expression in these cells. HCR mRNA expression was found in human astrocytes that was upregulated by pre-stimulation of the astrocytes with nitric oxide (NO), but not with lipopolysaccharide (LPS). Staining with a monoclonal antibody for HCR showed that HCR immunoreactivity was strongly localized in the astrocyte membranes. In addition, HEK293 cells that were transfected with the HCR gene and LPS-stimulated human monocytes also showed strong HCR protein expression in their cellular membranes. Thus, we assume that the HCR gene encodes for a membrane bound chemokine receptor. Functional studies in the HCR transfected HEK293 cells showed cellular migration and increases in intracellular calcium after stimulation of the cells with CCL2, CCL5, CCL7 and CCL8, but not with other chemokines tested. Finally, CCL2 stimulated HCR-transfected HEK293 cells showed a rapid and strong redistribution of filamentous actin to a polar distribution, thus showing a property of migrating cells (30). Evidently, this suggests that HCR is a functional chemokine receptor. Activation of chemokine receptors has been shown to induce a variety of signaling cascades including increases in intracellular calcium (see for instance 31). HCR transfected HEK293 cells did only respond with a detectable increase in calcium to a relatively high dose of chemokine, but showed migration to lower doses of the same chemokine. Our failure to detect an increase in intracellular calcium in response to chemokines in human astrocytes (data not shown) might be explained by the much lower expression of HCR in human astrocytes compared to HCR over-expressing HEK293 cells. The properties of the HCR receptor show strong resemblance to the properties of L-CCR. L-CCR and HCR protein sequences are highly homologous; L-CCR and HCR are expressed in mouse and human astrocytes, respectively. L-CCR and HCR expression in astrocytes is increased by treatment with proinflammatory stimuli and HEK293 cells transfected with L-CCR or HCR respond to similar chemokines with migration and intracellular calcium increases. Based on the current results we suggest that HCR is the human homologue of L-CCR.

The only observed difference between HCR and L-CCR was the failure of LPS to induce HCR expression in astrocytes. However, LPS induced effects on gene expression in human astrocytes is a matter of controversy (5, 21, 22, 33). Moreover, in our study HCR mRNA expression increased in human astrocytes after stimulation with NO. Thus, HCR is still a chemokine receptor that is induced under proinflammatory conditions.

Chemokine receptor activation has dramatic effects on the actin cytoskeleton arrangement. In resting astrocytes in culture, long parallel F-actin stress fibers are visible that may extend to multiple astrocytes (34). In contrast, freshly plated astrocytes show a typical rimlike organization of F-actin along their membranes (17). In time, these astrocytes will develop spreading and elaboration of actin cytoskeletal processes, with pronounced presence of parallel stress fibers after 24h (28). When we stimulated resting astrocytes with CCL2 the F-actin stress fiber arrangement was dramatically altered. The astrocytes showed rapid actin reorganization to a rimlike F-actin structure in response to CCL2. Although most chemokine receptors can be activated by multiple chemokines and one chemokine can activate a number of chemokine receptor subtypes no other chemokine receptors apart from CCR2 are known to be activated by CCL2. Furthermore, human astrocytes have previously been reported to respond to CCL2 (19), produce and release CCL2 (32), but CCR2 expression on human astrocytes is controversial (2, 14, 19). We did not detect CCR2 expression in post-mortem adult human astrocytes. Thus, our observation of dramatic F-actin rearrangement in astrocytes stimulated with CCL2 are indicative for the existence of another functional chemokine receptor. We suggest HCR is an alternative CCL2 chemokine receptor in human astrocytes.

That glial chemokine receptors are functional has been shown for a variety of different chemokine receptors (15). Their glial expression suggests that chemokines may contribute to an endogenous inflammatory cascade in the central nervous system, which is related to pathological conditions (9, 20). Since astrocytes are closely related to the blood brain barrier, it has been suggested that astrocytes have a role in leukocyte infiltration during brain inflammation (6). Expression and activation of chemokine receptors on astrocytes may be of great influence on the infiltration mechanism. Furthermore, the profile of chemokine expression and chemokine receptor expression in brain cells such as astrocytes depend on local circumstances. Characterization of chemokine receptors in astrocytes is therefore of great importance for our understanding of the role of chemokines in the central nervous system. For the first time, we show that the orphan chemokine receptor HCR is expressed in cultured human astrocytes and microglia, and might be a functional receptor for CCL2, indicating a role for HCR in the inflammatory response of the brain.

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